PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/53, 15/82, 15/52, 9/04, C12Q 1/68, 1/32, C12P 17/04, A01H 5/00

A1

(11) International Publication Number: **WO 99/33995**

(43) International Publication Date: 8 July 1999 (08.07.99)

(21) International Application Number: PCT/GB98/03903

(22) International Filing Date: 23 December 1998 (23.12.98)

(30) Priority Data:

9727255.3	23 December 1997 (23.12.97)	GB
9807358.8	7 April 1998 (07.04.98)	GB
9807360.4	7 April 1998 (07.04.98)	GB

(71) Applicants *(for all designated States except US)*: ASCORBEX LIMITED [GB/GB]; Reed Hall, University of Exeter, Exeter, Devon EX4 4QR (GB). BIOTECHNOLOGY SOUTH-WEST LIMITED [GB/GB]; Reed Hall, University of Exeter, Devon EX4 4QR (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: SMIRNOFF, Nick [GB/GB]; Ascorbex Limited, Reed Hall, University of Exeter, Exeter, Devon EX4 4QR (GB). WHEELER, Glen [GB/GB]; Ascorbex Limited, Reed Hall, University of Exeter, Exeter, Devon EX4 4QR (GB).

(74) Agents: DEAN, John, Paul et al.; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2QP (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PLANT GALACTOSE DEHYDROGENASE

(57) Abstract

This invention relates to an enzyme, L–galactose dehydrogenase, in particular the invention relates to an enzyme which catalyses the conversion of L–galactose to L–galactonolactone.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

PLANT GALACTOSE DEHYDROGENASE

This invention relates to a previously unknown enzyme and its uses and in particular, though not exclusively, to an enzyme which catalyses the oxidation of L-galactose to L-galactonolactone in the pathway producing L-ascorbic acid (L-threo-2-hexenono-1,4-lactone) in plants. This invention also relates to inhibitors or inactivators of such an enzyme, and to the production of transgenic plants with upregulated pathways producing L-ascorbic acid.

L-ascorbic acid ("ascorbate"), commonly known as vitamin C, is a major metabolite in plants reaching concentrations of 1-5 mM in leaves and up to 25 mM in chloroplasts. It functions as an antioxidant and has putative roles in photosynthesis and transmembrane electron transport as well as in protecting plants against a variety of stressful conditions such as air pollutants, temperature extremes and drought. It has also been suggested that L-ascorbic acid plays a role in cell expansion and cell division.

Plant-derived L-ascorbic acid is also the major source of vitamin C in the diet of humans and some animals (including other primates and guinea pigs), since they are unable to synthesise this essential antioxidant themselves. A lack of L-ascorbic acid in the diet can lead to collagen deficiency which results in the symptoms of scurvy. Sub-clinical scurvy is now a problem, particularly amongst those elderly who do not have a proper diet.

Although the complete biosynthetic pathway of L-ascorbic acid in animals has been well known for a considerable time, it has remained unknown in plants, despite its importance and widespread distribution.

The most effective precursor of L-ascorbic acid in plants is L-galactono-l,4-lactone. A mitochondrial enzyme, L-galactono-1,4-lactone dehydrogenase has been isolated from a variety of plants (which facilitate this conversion) (Mapson, L. W. & Breslow, *Biochem. J.* **68**, 395-406 (1958) and Oba, K., *et al J. Biochem.* **117**, 120-124 (1995)). However the role of this compound as the physiological precursor of ascorbate has been disputed. It has not been detected in plants and, more significantly, its proposed production from D--galacturonic acid (Mapson L. W. *et al. Biochem. J.* **64**, 13-22 (1956)) would require inversion of the hexose carbon skeleton, which extensive radio label tracer studies have

demonstrated does not occur during ascorbate synthesis from glucose (Loewus, F.A. *Phytochemistry* 2, 109-128 (1963)). However, if plants could produce L-galactono-1,4-lactone without inversion of the carbon chain, evidence for its involvement as a precursor in ascorbate biosynthesis would be strong.

The inventors have unexpectedly determined that the production of L-ascorbate in plants occurs via an interconversion of the sugar nucleotides GDP-D-mannose and GDP-L-galactose. D-mannose and L-galactose have been found to be efficient precursors of cellular ascorbate in plants. The inventors have also purified a novel enzyme which catalyses the oxidation of L-galactose to L-galactonolactone without inversion of the carbon chain, and have subsequently demonstrated the ability of plants to convert GDP-D-mannose to L-galactose and L-galactonolactone. The newly-discovered pathway by which plants synthesise L-ascorbic acid is shown in Fig. 5. The GDP-sugar derivatives (GDP-D-mannose and GDP-L-galactose) are utilised by plants in the synthesis of cell wall polysaccharides and glycoproteins.

Now the pathway has been elucidated, the opportunity exists to develop transgenic plants capable of the overproduction of L-ascorbic acid both to improve crop yield under stressful conditions, and also to improve the nutritional quality of fruit and vegetables.

The production of transgenic higher organisms would provide food with enhanced levels of vitamin C for human consumption.

These new sources of L-ascorbate will aid the prevention of scurvy in both the elderly and the third world populations where currently scurvy is a problem due to malnutrition.

In addition, the increased availability of L-ascorbate will allow further study of potential medical applications of the compound as well as provide an increased level of high quality, low cost L-ascorbate for use as vitamin C supplements to augment the human diet.

Further, it will be possible to create transgenic microorganisms for large scale production of L-ascorbate by fermentation for purification and commercial exploitation.

Further, the determination of the pathway, leading to the production of L-ascorbate in plants, by the inventors, has lead to the development of a method to increase the ascorbate

content of plant tissues involving the decreased expression of enzymes which divert carbon from the ascorbate biosynthesis pathway. Using this principle, the skilled person can develope transgenic organisms with increased levels of ascorbate.

In addition, the inventors have developed novel compounds which act as plant herbicides by inhibiting or inactivating the L-galactose dehydrogenase enzyme. Herbicides are compounds which kill or injure plants by a variety of mechanisms and are used in agriculture and horticulture to control weeds. This invention provides a new class of herbicides of great utility by virtue of their low toxicity to animals. Chemical compounds which inhibit or inactivate L-galactose dehydrogenase can be synthesised and used as herbicides. The inventors have also developed transgenic plants which exhibit resistance to such herbicidal action.

A first aspect of the invention provides an isolated enzyme L galactose dehydrogenase (formal name: L-galactono-1,4-lactone dehydrogenase (L-galactose:NADP(P)$^+$ oxidoreductase). This aspect of the invention also provides a polypeptide which comprises at least a portion of the amino acid sequence of SEQ 1, or equivalents to that sequence, or to portions of that sequence, which catalyse the conversion of L-galactose to L-galatonolactone by virtue of the degeneracy of the genetic code.

The term 'polypeptide' used herein embraces entities described in the literature and familiar to the skilled addressee as, for example, proteins, polypeptides and peptides.

Preferably, the polypeptide is isolated L-galactose dehydrogenase.

Preferably, the L-galactose dehydrogenase is NAD(P)-dependent.

Preferably the catalytic conversion is an oxidation reaction.

Preferably the oxidation reaction involves oxidation at C1.

Preferably, the conversion occurs in plants.

A second aspect of the invention provides a DNA sequence encoding a polypeptide according to a first aspect of the invention.

A third aspect of the invention provides an organism engineered to express the polypeptide according to a first aspect of the invention, by incorporation of the DNA sequence according to a second aspect of the invention within a gene expression control system.

Preferably, the whole or part of the organism overexpresses the polypeptide.

Preferably, the organism is a plant.The term "plant" used herein includes algae. In particular, preferably, the plant is selected from *Arabidopsis thaliana, Lycopersicon esculentum, Lycopersicon tuberosum.*

Alternatively, the organism is a bacterium, or a fungi such as a yeast, especially *Saccharomyces* species, or an animal. Where the organism is an animal, it is preferably a mammal.

Preferably, the DNA sequence encodes a polypeptide conferring increased tolerance to environmental stresses including those causing oxidative stress to an organism engineered to express that polypeptide.

A fourth aspect of the invention provides a probe wherein the probe comprises at least a portion of a polypeptide according to a first aspect of the invention. The probe may be used to locate similar enzymes or the same enzyme in other species.

A fifth aspect of the invention provides a probe comprising at least a portion of the DNA sequence according to the second aspect of the invention, and equivalents to that sequence or to portions of that sequence, which encode a polypeptide according to a first aspect of the invention. The probe may also comprise RNA equivalent to such a DNA sequence. Either nucleotide probe may be used to locate similar enzymes ot the same enzyme in other species.

A sixth aspect of the invention provides the production of the polypeptide according to a first aspect of the invention by chemical or biological means.

An seventh aspect of the invention provides diagnostic tests, assays, or monitoring methods using all or part of a polypeptide according to a first aspect of the invention.

An eighth aspect of the invention provides diagnostic tests, assays, or monitoring methods using a probe according to a fourth and fifth aspect of the invention.

Preferably, the diagnostic tests, assays, or monitoring methods comprise microbiological, animal cell or biodiagnostic tests, assays and monitoring methods. The biodiagnostic tests include assays using whole or partial cell extracts or other fluids such as urine or saliva where intact cells are not involved, e.g a specific assay for L-galactose.

A ninth aspect of the invention provides a multi-enzyme pathway, or method of producing L-ascorbic acid or a precursor thereof, wherein one of the steps of the pathway is catalysed by L-galactose dehydrogenase or a polypeptide according to a first aspect of the invention.

Preferably, the pathway includes any or all of the enzymes :hexokinase, glucose (hexose)-phospate isomerase, phosphomannose isomerase,phosphomannose mutase,GDP-D-mannose pyrophosphorylase, GDP-D-mannose-3,5-epimerase, GDP L-galactose pyrophosphorylase, GDP-L-galactose phosphorylase, L-galactose-1-phosphate phosphatase, L-galactose dehydrogenase, and L galactono 1-4-lactone dehydrogenase. The pathway may provided *in vitro* or *in vivo*.

Any of these intermediates, in particular GDP-D-mannose or GDP-L-galactose may be a branch point for other multienzyme pathways with diverse end products.

A tenth aspect of the invention provides ascorbic acid produced by a pathway according to a ninth aspect of the invention.

A eleventh aspect of the invention provides a compound produced as an intermediate of a pathway according to a ninth aspect of the invention.

A twelfth aspect of the invention provides a compound obtained from a precursor compound wherein the precursor compound is produced by a pathway according to a ninth aspect of the invention.

A thirteenth aspect of the invention provides a dietary supplement comprising ascorbic acid according to an eleventh aspect of the invention.

A fourteenth aspect of the invention provides an organism wherein the whole or any part of the organism contains increased levels of ascorbic acid according to an eleventh aspect of the invention.

Preferably the organism is a plant.

Preferably the plant includes *Arabidopsis thaliana, Lycopersicon esculentum* and *Lycopersicon tuberosum* and *Saccharomyces* species.

The invention will now be described, by way of example only, with reference to SEQ 1 and to further Figs. 1 to 4 and 6 to 7, in which:

SEQ 1 shows the partial polypeptide sequence of L-galactose dehydrogenase from pea seedlings (*Pisum sativum*).

Fig 1. is a graph showing ascorbate production in barley leaf segments exposed to different substrates;

Fig. 2 is a graph, showing the kinetic characteristics of L-galactose dehydrogenase partially purified from pea embryonic axes by ammonium sulphate precipitation and hydrophobic interaction chromatography;

Fig. 3 is a series of radiochromatograms showing the level of radiolabel incorporation into carbohydrates;

Fig. 4 is a graph showing the level of radioactivity in ascorbic acid recovered from *A. thaliana* leaves;

Fig. 6 is a set of graphs showing the inhibitory effect of L-galactano-1,4-lactone on L-galactose dehydrogenase; and

Fig. 7 shows an SDS-PAGE analysis of 1 ml fractions eluting from a Superose 12 gel filtration column.

1. Production of L-galactono-1,4-lactone in plants

To investigate the possible source of galactono-lactone in plants which would not involve inversion of the carbon chain and would therefore implicate L-galactono-1,4-lactone dehydrogenase in ascorbate synthesis, L-galactose was supplied to slices of primary leaves of 1 week old barley seedlings (*Hordeum vulgare* cv. Golden Promise). This resulted in a rapid and substantial increase in the foliar ascorbate concentration, similar to that induced by L-galactono-1,4-lactone (Fig. 1). L-galactose feeding also dramatically increased ascorbate concentration in *Arabidopsis thaliana* leaves and in embryonic axes of germinating pea seedlings (*Pisum sativum* cv. Meteor). The identity of the product as L-ascorbate was confirmed by its reactivity with ascorbate oxidase, its ability to reduce acidic DCPIP and by co-chromatography with L-ascorbate using TLC and HPLC (Chen, YT *et al, Biochem. J.*, **55**, 821-23 (1953); Conklin, PL *et al, Plant Physiol*, **115**, 1277-1285 (1997)). The inventors were able to demonstrate an accumulation of L-galactose after it was fed exogenously to pea embryonic axes. L-galactose was measured by gas chromatography of its trimethylsiyl derivative (Andrews, M.A. Carbohydrate Research **194**: 1-19 (1989) The L-galactose was undetectable one hour after removal of the external supply showing that it is rapidly and efficiently metabolised.

L-galactose is therefore an effective precursor of L-ascorbate in plants. Oxidation of L-galactose at C1 would result in the production of L-galactonolactone and the presence of this activity was investigated in cell-free extracts of *A. thaliana* leaves and pea embryonic axes. Both these supported L-galactose-dependent NAD and NADP reduction. The enzyme activity from pea embryonic axes has been purified as follows:

2. Purification of L-galactose dehydrogenase

L-Galactose dehydrogenase was extracted from shoots of pea seedlings which had been germinated in the dark for 6 days. The shoots were homogenised in 50 mM Tris-HCl pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT) and 5% (v/v) glycerol (0.5g shoots/ml). The homogenate was strained through cheesecloth and then centrifuged at 26,000 g for 20 minutes at 4°C. The supernatant was brought to 50% saturation with ammonium sulphate (29.1g/100ml) and centrifuged as before. The

supernatant was then brought to 70 % saturation with ammonium sulphate (12.5g/100 ml) and centrifuged as before. The pellet, which contained L-galactose dehydrogenase activity, was resuspended in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 2 mM DTT and 20% (v/v) glycerol.

The sample was loaded onto a chromatography column (300 x 7 mm) packed with Phenyl Sepharose® (Pharmacia) previously equilibrated with 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT and 1 M $(NH_4)_2 SO_4$. The proteins were eluted with a gradient of 1-0 M $(NH_4)_2 SO_4$ in equilibration buffer and those fractions containing L-galactose dehydrogenase activity were pooled. This sample was then dialysed for 16 h against 25 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM DTT and then loaded onto a column (650 x 16 mm) packed with an anion exchange gel (DEAE-Sephacel®, Pharmacia) previously equilibrated with 25 mM Tris-HCl pH 8.5, 1 mM EDTA and 1 mM DTT. The column was eluted with a gradient of KCl (0 - 0.6 M) in the equilibration buffer. Fractions containing L-galactose dehydrogenase activity were pooled. This sample was then dialysed for 16 h against 25 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM DTT. The dialysed sample was then loaded onto a column (300 x 17 mm) packed with Cibacron Blue Sepharose® (Pharmacia), previously equilibrated with 25 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM DTT. The column was washed with a gradient of 10 mM nicotinamide adenine dinucleotide (NAD) in 25 mM Tris-HCl pH 7.5 - 10 mM NAD in 25 mM Tris-HCl pH 8.5. The latter buffer eluted L-galactose dehydrogenase activity. The active fractions were then concentrated by centrifugation through a membrane (Centricon 30, Amicon Corporation). The concentrated sample was loaded onto a gel filtration column (Superose 12®, 450 x 25mm, Pharmacia) previously equilibrated with 25 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM DTT. The proteins were eluted with the same buffer. The volume of buffer required to elute L-galactose dehydrogenase activity was determined. This volume was compared with the elution volumes of standard proteins of known molecular mass. From this comparison the relative molecular mass (Mr) of L-galactose dehydrogenase was determined to be 152,000.

The fractions from the gel filtration column containing L-galactose dehydrogenase activity were then concentrated by adding acetone to the sample to give a final acetone

concentration of 80% (v/v). After leaving at -20°C for 1 h the precipitated protein was pelleted by centrifugation at 12,000g for 10 minutes. The protein was then redissolved in water and mixed with an equal volume of double-strength Laemmli buffer (Hames and Rickwood, 1990) and boiled for 3 minutes. The denatured protein was then subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This was done by loading 10 µl samples, prepared as above, onto a gel prepared according to standard procedures (Hames and Rickwood, 1990). The concentration of acrylamide in the gels was 10%. The samples were subject to electrophoresis at 50 volts for 15 minutes and followed by 100 volts until the bromophenol blue marker reached the end of the gel. At this point the proteins were visualised by staining with coomassie blue (Hames, B.D. and Rickwood, D. (1990). *Gel Electrophoresis of Proteins. A Practical Approach.* IRL Press/Oxford University Press, Oxford.) or silver (Bio-Rad Silver Stain Plus, procedure according to manufacturer's instructions). The Mr of the denatured polypeptides was determined by comparing their migration distance with protein standards of known Mr. It was determined that a polypeptide of Mr 39,800 was present in the fractions from the gel filtration column which contained L-galactose dehydrogenase activity. This was quantitatively the major protein in these fractions and its amount, determined by intensity of staining, varied in exact proportion to L-galactose dehydrogenase activity (See Fig. 7A, B and C). In Fig. 7:
a) The gel displays a major band at 39.8kD which coincides with the L-galactose dehydrogenase activity of the fractions. Activities are 0, 6, 17, 23.4, 27.8, 6.99 and 0 mAbs/min for samples a-f which eluted after 45, 49, 52, 57, 64 and 70 ml respectively.

b) Calibration curve based on migration of five proteins standards in left hand lane. Standards are lysozyme (14.4kD), soybean trypsin inhibitor (21.5), carbonic anhydrase (31), ovalbumin (45) and bovine serum albumin (66.2).

c) Quantitation of protein size for bands 1-3, corresponding to fractions b-d, based upon band migration relative to protein standards.

No other polypeptides followed this pattern. It is concluded that this polypeptide is a subunit of L-galactose dehydrogenase. L-galactose dehydrogenase is therefore a homotetrameric protein of Mr 152,000 consisting of 4 subunits each having an Mr 39,800.

3. Determination of the N-terminal sequence of pea L-galactose dehydrogenase

A sample of L-galactose dehydrogenase, purified as described above, was precipitated with 80% (v/v) acetone and then redissolved in 0.1 ml of 0.1% mM trifluoroacetic acid. The sample was applied to a ProSorb™ sample preparation device according to the manufacturer's instructions (Perkin Elmer Applied Biosystems, Foster City, California, USA). Automated sequencing, using the Edman degradation method (Findlay and Geishow, 1989), was carried out, according to the manufacturer's instructions, with an Applied Biosystems Model 477A protein sequencing instrument equipped with on-line analysis of 3-phenyl-2-thiohydantoin (PTH) amino acid derivatives. These were monitored at 269 nm after separation on a C-18 reversed phase high performance liquid chromatography column. This method detected the sequence of the first nineteen amino acids of L-galactose dehydrogenase, starting at the amino terminus of the protein. The sequence is shown in SEQ 1.

A BLASTP search was carried out using the partial peptide sequence obtained (Altshul, Stephen F. *et al*, (1997), *Nucleic Acid Res.* **25**: 3389-3402). The partial peptide sequence was found to have 72 % identity with an *Arabidopsis thaliana* putative peptide sequence of unknown function (Accession No. 3549669). The 72 % identity was observed in respect of amino acids 5 to 22 of the *Arabidopsis thaliana* sequence.

Due to the level of sequence identity, it is likely that this *Arabidopsis thaliana* putative protein is, in fact, an L-galatose dehydrogenase enzyme.

4. Determination of enzyme activity

The enzyme was assayed for L-galactose dehydrogenase activity by following NAD(P)H formation at 340 nm in 50 mM tris-HCL buffer, at pH 7.5 with 0.1 mM NAD(P) and various L-galactose concentrations. The enzyme was shown to have a preference for NAD and shows very little activity with a range of other sugars (D-galactose, D-glucose,

D-mannose, L-fucose and D-arabinose). The identity of the reaction product was determined by a lactone assay and gas chromatography-mass spectrometry (GC-MS) as follows:

The reaction product from the L-galactose dehydrogenase assay was adjusted to pH 9.5 with NaOH solution and left for 2h at room temperature to delactonize any aldonolactones and uronolactones present. It was then loaded onto a column of Dowex 1-formate (anion exchange resin). The column was eluted sequentially with water and 0.1 M formic acid. The formic acid fraction was heated at 100°C for 10 minutes and then dried under an air stream to relactonize any aldonic or uronic acids present. The dried samples were then coverted to trimethylsilyl (TMS) derivatives (Andrews, 1989 *supra*). The derivatives were separated by capillary gas chromatography (GC) on a Hewlett Packard Ultra 2 column (50 m, 0.37 mm i.d., crosslinked 5% Phe Me silicone, 0.17 :m film thickness) and detected with a flame ionization detector. The temperature programme was 145 °C for 10 min, increased to 170°C at 5° min^{-1}, held for 10 min, then increased to 305°C at 25° min^{-1} and held for 5 min. The reaction product produced one peak with identical relative retention time to pure L-galactono-1,4-lactone (using D-arabitol as the internal standard). The same TMS derivatives were analysed by gas chromatography-mass spectrometry (GC-MS) on a Hewlett Packard Ultra 1 column (50 m, 0.2 mm i.d., crosslinked Me silicone, 0.33 μm film thickness) using the same temperature programme as GC. The resulting mass spectrum of the product was identical to L-galactono-1,4-lactone. The product from the reaction mixture was quantified using an assay based on the reaction of hydroxylamine with lactones followed by formation of a coloured complex with ferric iron (Kim S-T *et al.*, *Biochem Biophys Acta*, **1297**, 1-8 (1996).

The product from the reaction mixture showed a positive response with the hydroxylamine assay for lactones, with approximately one to one stoichiometry between NADH formation and lactone production (in L-galactono-1,4-lactone equivalents).

It is likely that the product was initially L-galactono-1 ,5-lactone which is relatively unstable and spontaneously converts to the 1,4 form. The K_m for L-galactose was 0.3 mM (Fig. 2). The enzyme was able to oxidise L-sorbosone, another suggested intermediate in

plant ascorbate biosynthesis (Saito, K, *et al. Plant Physiol.* **94**, 1496-1500 (1990)), but with very low affinity (Fig. 2). It is suggested that the product would be L-ascorbic acid, which could explain why labelled L-sorbosone is converted to ascorbate (Saito, K, *et al. Plant Physiol.* **94**, 1496-1500 (1990)). L-Sorbosone has the same configuration as L-galactose, apart from the keto group at C2 and hydroxyl group at C3.

Therefore, the inventors have discovered a novel higher plant enzyme (L-galactose dehydrogenase) capable of forming L-galactono-l, 4-lactone from L-galactose with high affinity and specificity.

Other known dehydrogenases which oxidise C1 of non-phosphorylated aldoses are: D-galactose dehydrogenase in *Pseudomonas fluorescens* (Maier, E. & Kurtz, G. D *Methods in Enzymology* **89**, 176-181 (1982)); L-fucose dehydrogenase in mammals (Schachter, H. *et al J. Biol. Chem* **244**, 4785-4792 (1969)) and D-arabinose dehydrogenase in *Candida albicans* (Kim S-T *et al Biochem. Biophys Acta*, **1297**, 1-8 (1996)).

L-galactose occurs in non-cellulosic cell wall polymers indicating that plants are able to synthesise this compound (Baydoun EAH & Fry SC, *J. Plant Physiol. 132 484-490 (1988)).* The L-galactose residues in the cell wall are derived from GDP-L-galactose, a nucleotide sugar formed via GDP-D-mannose-3,-5-epimerase activity, which has been reported in *Chlorella* (Barber, G. A. *J. Biol. Chem.* **254**, 7600-7603 (1979)) and a number of higher plants (Barber, G. A. *Arch. Biochem Biophys*, **147**, 619-623 (1971)).

5. Detection of GDP-D-mannose-3,5-epimerase activity

GDP-D-mannose-3,5-epimerase activity was detected by homogenisation of tissue in 100 mM tris-HCl buffer pH 7.6 containing 5 mM DTT, 1 mM EDTA and 1 % polyvinylpolypyrrolidone before centrifugation at 5000 rpm (12,000g) for 20 minutes. The supernatant was then either used directly as a crude extract or precipitated with 90% saturation ammonium sulphate and then desalted with Sephadex G-25 (Pharmacia PD10 column). Crude extracts or precipitates were incubated with GDP-D-[U^{14}C]mannose in 25mM Tris-HCl pH 8.0 containing 2mM EDTA at 25°C. The reaction products were passed through a strong anion exchange (SAX) column. Neutral fractions were deionised with a strong cation exchange (SCX) column prior to thin layer chromatography (TLC).

GDP-sugars were eluted from the SAX column with 2M formic acid and then hydrolysed with 2M trifluoroacetic acid at 80° C for 1 hour prior to TLC to produce free carbohydrates. Carbohydrates were separated by TLC on silica plates (Whatman) impregnated with 0.3M sodium dihydrogen orthophosphate, using an acetone/butanol/water (8:1:1 v/v) solvent (Ghebreqzabher M. *et al. J. Chromatogr.*, **127**, 133-162 (1976)). TLC plates were scanned with a Linear Analyser made by Berthold to detect radioactivity. Radioactive peaks were identified by their co-chromatography with authentic standards detected by an aniline/diphenylamine stain (Dawson RMC *et al. Data for Biochemical Research.* Clarendon Press. Oxford (1969) 3nd ed.). D- and L-galactose are not resolved, so the identity of the reaction product was confirmed using D-galactose dehydrogenase to selectively remove this compound (Roberts R. M & Harper E. *Phytochemistry*, **12**, 2679-2682 (1986)).

6. L-galactose production

The inventors were able to detect GDP-D-mannose-3,5-epimerase activity using both crude extracts and protein precipitates from pea embryonic axes (Fig 3) and protein precipitates from *A. thaliana* leaves. However free L-galactose would be necessary for L-galactose dehydrogenase. Free L-galactose has not been detected in plant extracts, although the majority of chromatographic techniques do not differentiate between the D- and L isomers of this sugar. Nevertheless it has been possible to demonstrate the substantial production of free L-galactose from the crude extracts of pea embryonic axes (Fig. 3). Since free D--mannose was not formed in appreciable quantities (Fig. 3), it is likely that this hydrolysis was not the result of non-specific phosphatase or pyrophosphorylase activity in the extracts. The addition of NAD to the extracts during the incubation period resulted in the total loss of L-galactose from the neutral sugar fraction. A significant peak was identified in these fractions which co-eluted with L-galactonolactone (Fig 3), suggesting that these extracts have retained L-galactose dehydrogenase activity also.

The results suggest plants possess the ability to synthesise ascorbate via GDP mannose. To confirm whether this process occurred *in vivo*, radio labelled D-mannose was fed to *A. thaliana* leaves via the transpiration stream. Tissue extracts were then prepared and fractionated by ion exchange chromatography as set out below.

7. Determination of radioactive ascorbate content in leaves fed radiolabelled precursors.

Leaf tissue which had been fed the radiolabelled precursor via the petiole was frozen at -70°C immediately after the incubation period. Leaf tissue was then ground to powder in a mortar and pestle containing liquid nitrogen. Before the powder thawed, 1ml of 5% perchloric acid containing 1mM EDTA was added and the tissue was thoroughly homogenised. The extract was then centrifuged at 12,000g. for 2 minutes and the supernatant collected. 2.5 µl of methyl orange were added as an indicator and the solution was neutralised with the addition of 5M KCO_3 (approx 60 µl). The extract was allowed to stand for 10 minutes before centrifugation at 12000g. for 1 minute. The supernatant was collected, mixed with an equal volume of 10% dithiothreitol and allowed to stand for 10 minutes to facilitate the reduction of any oxidised ascorbate.

The extract was then passed through a SAX column to which acidic compounds bind (such as ascorbate). The column was then rinsed with 5ml of deionised water before the ascorbic acid was eluted using 5ml of 60mM formic acid. Any further acidic compounds were later eluted using 5ml of 2M formic acid. The 60mM fraction was frozen immediately and freeze dried. The ascorbic acid was then reconstituted in 200 µl of 10mM formic acid and 100 µl was injected onto the HPLC system. Ascorbic acid was detected by its UV absorbance at 210nm. Radioactivity in ascorbic acid was measured by fraction collection of the eluent followed by liquid scintillation counting. Fractions of the HPLC eluent were collected every 0.2 minutes and to each of these 4ml of scintillation fluid (Emulsifier Safe) were added. The radioactivity in each was measured using a liquid scintillation counter. The total radioactivity in those fractions corresponding to the ascorbate peak from the UV detector was then used to calculate the total amount of radioactive ascorbate in the leaf tissue.

The effective incorporation of exogenously fed D-mannose into cell wall components has led to the suggestion that D-mannose is not extensively metabolised via the main glycolytic pathway in plants and is instead utilised in the production of nucleotide sugar intermediates (Roberts RM *Arch. Biochem. Biophys.* **145**, 685-692 (1971)). A significant incorporation of radio label from D-mannose into ascorbate would therefore be expected. The level of

incorporation of radio label in ascorbate is detected by purification of the ascorbate by ion exchange fractionation followed by HPLC (Conklin P. L *et al. Plant Physiol.* **115**, 1277-85 (1997)). Fig. 4 shows the radioactivity recovered in ascorbic acid from *A. thaliana* leaves after the petiolar feeding of [U-^{14}C]-D-glucose (open symbols) and [U-^{14}C]-D-mannose (closed symbols) for 4 hours. The graph indicates the proportion of total radioactivity present in the eluent fractions collected during HPLC analysis of ascorbate. The retention time of non-labelled ascorbate is indicated with an arrow. The significant peak eluting prior to ascorbate is thought to be dehydroascorbate. The proportion of radio label from D-glucose incorporated into ascorbate is usually low, around 1% or less. In *A. thaliana* leaves supplied with D-[U-^{14}C]mannose, incorporation of label into ascorbate was found to be 10% (Fig. 4). The same result was found with marrow roots (data not shown). This is a clear indication of the effective conversion of D-mannose to ascorbate. High concentrations of exogenous mannose did not affect ascorbate pool size suggesting that its conversion to L-galactose is a rate limiting step since L-galactose is known to be readily phosphorylated to mannose-6-phosphate (Harris G. C *et al. Plant Physiol.* **82**, 1081-89 (1986)).

8. Genetically Modified Plants capable of elevated levels of expression of the enzyme L-galactose dehydrogenase or other pathway enzymes, and capable of synthesising enhanced levels of ascorbic acid.

These results have allowed the proposal of a pathway for ascorbate biosynthesis in higher plants which is consistent with all previous evidence (Fig. 5). The ability of plant extracts to catalyse all the proposed reactions has been demonstrated, and a novel enzyme, L-galactose dehydrogenase, has been detected. The pathway reconciles the apparent contradiction between the ease of oxidation of L- galactono- 1 ,4-lactone to ascorbate and the previous labelling data. The proposed pathway, in which L-galactono-1,4-lactone is produced from D-glucose via D-mannose and L-galactose, is in accordance with Loewus' statement that in the conversion of D-glucose to ascorbate in plants, there is no inversion of the carbon chain, there is conservation of the hydroxymethyl group at C6 and there is an epimerization at C5 (Grhn, M., *et al Pelargonium crispum* L. L'Her. *Plant Physiol.* **70**, 1233 (1982)). The ability of plants to incorporate label from the osones (D-glucosone and L-sorbosone) into ascorbate is also in accordance with this scheme (Saito K., *et al. Plant Physiol.* ***94***,1496-1500 (1990)) and have demonstrated that L sorbosone is a substrate

analogue for L-galactose dehydrogenase (Fig. 2). The very low affinity "L- sorbosone dehydrogenase" activity previously reported (Loewus, M. W., *et al. Plant Physiol.* **94**, 1492-1495 (1990)) could therefore be L-galactose dehydrogenase.

An alternative approach to increase the ascorbate concentration in plant tissues involves decreasing the activity of enzymes which catalyse reactions that branch from the pathway and lead to alternative products. This is done by producing transgenic plants which express the genes encoding these enzymes in antisense orientation, using techniques well-known to those experienced in the art (Miki, I.L.A. and Iyer, V.N (1997). In: Plant Metabolism, ed. Dennis, D.H. *et al.*, pp. 561-579, Longman).

In a further approach to increase the ascorbate production in microorganisms (including microalgae, bacteria and yeast), the activity of enzymes which catalyse reactions that in the ascorbate pathway is increased and/or the activity of enzymes which catalyse reactions that branch from the ascorbate pathway is decreased to produce significantly high yields of ascorbate by fermentation methods. Such modulations in the activity of enzymes is achieved by the use of microorganisms which are genetically modified. As used herein, a genetically modified microorganism, such as *Escherichia coli*, has a genome which is modified (i.e., mutated or changed) from its normal (i.e., wild- type or naturally occurring) form. Genetic modification of a microorganism can be accomplished using classical strain development and/or molecular genetic techniques. Such techniques are generally disclosed, for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press. The reference Sambrook et al., *ibid.*, is incorporated by reference herein in its entirety. Additionally, techniques for genetic modification of a microorganism are described in detail in the literature. A genetically modified microorganism can include a natural genetic variant as well as a microorganism in which nucleic acid molecules have been inserted, deleted or modified (i.e., mutated; e.g., by insertion, deletion, substitution, and/or inversion of nucleotides), in such a manner that such modifications provide the desired effect within the microorganism. According to the present invention, a genetically modified microorganism includes a microorganism that has been modified using recombinant technology. As used herein, genetic modifications which result in a decrease in gene expression, in the function of the gene, or in the function of the gene product (i.e., the protein encoded by the gene) can be referred to as inactivation

(complete or partial), deletion, interruption, blockage or down-regulation of a gene. For example, a genetic modification in a gene which results in a decrease in the function of the protein encoded by such gene, can be the result of a complete deletion of the gene (i.e., the gene does not exist, and therefore the protein does not exist), a mutation in the gene which results in incomplete or no translation of the protein (e.g., the protein is not expressed), or a mutation in the gene which decreases or abolishes the natural function of the protein (e.g., a protein is expressed which has decreased or no enzymatic activity or action). Genetic modifications which result in an increase in gene expression or function can be referred to as amplification, overproduction, overexpression, activation, enhancement, addition, or up-regulation of a gene.

In one embodiment of the present invention, a genetic modification of a microorganism increases the action of a protein involved in an ascorbate metabolic pathway or decreases the action of a protein involved in a pathway competing with the ascorbate metabolic pathway according to the present invention. Such a genetic modification includes any type of modification and specifically includes modifications made by recombinant technology and by classical mutagenesis. For example, in one embodiment, a microorganism of the present invention has a genetic modification that decreases the action of any enzyme catalyzing the conversion of GDP-D-mannose other than further along in the ascorbate pathway described herein. For example, in pathways competing with production of ascorbate, GDP-D-mannose is converted to GDP-D-mannuronic acid (by GDP-D-mannose dehydrogenase), GDP-D-rhamnose, GDP-L-fucose and other compounds which are precursors of polysaccharides. Reference is made herein to the literature on such competing pathways for identification of specific genes encoding enzymes catalyzing such reactions for modification as discussed herein. It should be noted that reference to decreasing the action (or activity) of an enzyme refers to any genetic modification in the microorganism in question which results in decreased functionality of the enzymes and includes higher activity of the enzymes (e.g., specific activity or *in vivo* enzymatic activity), increased inhibition or degradation of the enzymes and underexpression of the enzymes. For example, gene copy number can be decreased, expression levels can be decreased by use of a promoter that gives lower levels of expression than that of the native promoter, or a gene can be altered by genetic engineering or classical mutagenesis to decrease the action of an enzyme. For example, the action of an enzyme of the present invention can be

decreased by blocking or reducing the production of the enzyme, reducing enzyme activity, or inhibiting the activity of the enzyme. Blocking or reducing the production of an enzyme can include placing the gene encoding the enzyme under the control of a promoter that requires the presence of an inducing compound in the growth medium. By establishing conditions such that the inducer becomes depleted from the medium, the expression of the gene encoding the enzyme (and therefore, of enzyme synthesis) could be turned off. Blocking or reducing the activity of an enzyme could also include using an excision technology approach similar to that described in U.S. Patent No. 4,743,546, incorporated herein by reference. To use this approach, the gene encoding the enzyme of interest is cloned between specific genetic sequences that allow specific, controlled excision of the gene from the genome. Excision could be prompted by, for example, a shift in the cultivation temperature of the culture, as in U.S. Patent No. 4,743,546, or by some other physical or nutritional signal.

As another example, a microorganism of the present invention has a genetic modification that increases the action of any enzyme catalyzing the conversion of precursors to GDP-D-mannose and GDP-D-mannose further along in the ascorbate pathway described herein. For example, with reference to Fig. 5, the action of one or more of the six enzymes can be increased as discussed herein. Reference is made herein to the literature on such metabolic steps for identification of specific genes encoding enzymes catalyzing such reactions for modification as discussed herein.

9. Creation of transgenic plants

The gene encoding mannosyltransferase has previously been identified and cloned. This gene, contained in a vector in antisense orientation, can then beused to genetically transform a plant using established technology (Miki, I.L.A. and Iyer, V.N (1997). Plant Metabolism, ed. Dennis, D.H. *et al.*, pp. 561-579, Longman). The resulting plant has reduced mannosyltransferase activity, and therefore a higher level of diversion of GDP-mannose to ascorbic acid synthesis. Targeting expression of the antisense gene to specific tissues, for example fruits (using gene promotors known to drive gene expression in these specific tissues), will provide increased ascorbate concentration in these specific tissues. Dependent on the precise agronomic, organoleptic or nutritional characteristics

required, it is necessary to screen progenies for those plants exhibiting an optimally-balanced phenotype dependent in turn on the levels of antisense suppression achieved. A side effect of this genetic manipulation will be to alter the polysaccharide composition of the cell wall.

10. Use of plant enzymes as herbicide targets

Animals are well-known to produce L-ascorbic acid by a distinct biochemical pathway. Humans are unable to synthesise L-ascorbic acid. The unique role of L-galactose dehydrogenase in this pathway allows the inventors to develop herbicides comprising specific inhibitors or inactivators of plant L-galactose dehydrogenase. Such herbicides exhibit little toxicity to other organisms. The design of these compounds is likely, though not necessarily, to be based on knowledge of the structure of the natural substrate and/or on the structure of the catalytic site of the enzyme.

The gene encoding the enzyme of the invention can be cloned by using the peptide sequence information to design oligonucleotides which can be used to probe restricted genomic digests, followed by single colony blotting using a labelled oligonucleotide probe.

Furthermore, crop plants with resistance to such herbicides can be produced by modifying the DNA sequence of the gene encoding L-galactose dehydrogenase (for example, by using random or site-directed mutagenesis so that the modified enzyme is no longer inhibited by its specific herbicide. The modified gene is then introduced into any suitable crop plant by the standard techniques employed by those skilled in the art for production of transgenic plants. The resulting transgenic plant will be resistant to herbicides targeted to L-galactose dehydrogenase.

11. Generation of herbicide resistant plants

The identification of L-galactose dehydrogenase as a key enzyme in ascorbate biosynthesis has enabled the isolation, purification and *in vitro* assay of the enzyme as previously

described. The inventors have synthesised inhibitors which resembles the substrate (L-galactose) and which binds to the active site of L-galactose dehydrogenase. An example of such an inhibitor is L-galactono-1,4-lactone, the natural product of the reaction, which inhibits the activity of the enzyme in an uncompetitive manner (Fig. 6). Further chemical modification of this substrate, for example, by alkylation of the hydroxyl groups of carbon atoms 5 and 6, will prevent its further metabolism to L-ascorbic acid thereby increasing its efficiency as a herbicide. The same principle can be applied to any other compound which inhibits or inactivates L-galactose dehydrogenase. Potential inhibitors and inactivators of L-galactose dehydrogenase are detected by showing their effect on the activity of the purified or partially purified enzyme assayed *in vivo*.

L-Galactose dehydrogenase from pea embryonic axes is purified as previously described. The enzyme is assayed for L-galactose dehydrogenase activity by following NAD(P)H formation at 340 nm in 50 mM tris-HCL buffer, at pH 7.5 with 0.1 mM NAD(P).

A plant with resistance to herbicides targetted to L-galactose dehydrogenase can be produced by modifying the DNA sequence of the gene encoding L-galactose dehydrogenase (for example, by using random or site-directed mutagenesis (Directed Mutagenesis. A Practical Approach. Ed. M.J. McPherson, IRL Press, Oxford, 1991)) so that the modified enzyme is no longer inhibited by its specific herbicide. The modified gene is then introduced into, and expressed in, any suitable crop plant by the standard techniques employed by those skilled in the art (e.g. Filliati *et al,* Biotechnology **5,** 728-730 (1987) and McCormick *et al.,* Plant Cell Reports (1986) **5,** 81-84.) for production of transgenic plants. The resulting transgenic plant will be resistant to herbicides targeted to L-galactose dehydrogenase.

12. Increasing the levels of Ascorbic Acid in transgenic organisms

Identification of the ascorbate biosynthesis pathway fills a major gap in plant carbohydrate metabolism, since up to 10% of the soluble carbohydrate content of leaves comprises L-ascorbate. It is now possible to investigate the control of ascorbate biosynthesis in plants such as potatoes and tomatoes and to manipulate its content with potential benefits for

human nutrition and plant resistance to oxidative stress. Transgenic organisms can be created which have increased levels of ascorbic acid in their tissues.

This involves the insertion of the target gene sequence into genetic vectors capable of directing the expression, preferably at high levels, of the target polypeptide. Such a polypeptide would comprise the enzyme L-galactose dehydrogenase, or other ascorbic acid biosynthesis pathway enzymes.

In the case of plants, for the purposes of example, such a vector is commonly known as pPB1121. Another is pGPTV-kan. In the case of pPB1121 or pGPTV-kan which are each components of a so-called binary Agrobacterium vector system, the novel nucleotide coding sequence of the enzyme L-galactose dehydrogenase or other pathway enzymes may be inserted via prior excision of the beta glucuronidase reporter gene, such that the L-galactose dehydrogenase or other pathway enzymes gene becomes transcriptionally fused to a functional promoter gene, in this case the 35Spromoter of CaMV which will direct enzyme synthesis in most organs of the plant. Other promotors may also be used in place of 35S especially if tissue specific effects, for example high ascorbate tomato fruit, are required. Such genetic constructs are next transferred to the Agrobacterium component of the vector system. A preferred example of this is the Agrobacterium strain LBA4404 which is appropriate for the transformation of solanacious species like tomato and potato. In order to achieve this transfer, DNA of the genetic construct of pPBI121 or pGPTV-kan having been passaged and amplified in *E. coli*, is added to a 37°C culture of competent Agrobacterium cells prepared via preculture in calcium chloride, freezing in liquid nitrogen, holding at -70°C and thawing.

The ability to transfer the novel genetic construct to plants using the Agrobacterium strain constructed above is dependent upon a process known to those skilled in the art as co-cultivation, whereby the Agrobacterium strain is cultured *in situ* with tissue explants of the chosen plant species. Thereafter, the Agrobacterium is eliminated by treatment (counterselection) with an antibiotic, and transformed plant tissues are selected by means of a second antibiotic such as kanamycin which is lethal to untransformed tissues. Transformed plant tissues are induced to redifferentiate shoots and roots by established tissue culture methods depending on the plant species involved. Transformed plants thus

recovered from the TO generation are tested for ability to produce altered levels of enzyme L-galactose hydrogenase or other pathway enzymes. Such plants are then self pollinated to produce a T1 seed population which is segregating for the new trait. Individual plants grown from T1 seed which are null (azygous), monozygous or dizygous for the novel trait may be identified by conventional PCR methodology. Such plants are then used to demonstrate that monozygous or dizygous plants are capable of producing more ascorbic acid than the azygous control.

A detailed procedure is presented below for example of the Agrobacterium mediated transformation and recovery of tomato plants. The procedure is derived from those published by Filliati *et al*, Biotechnology **5** p728-730 (1987) and McCormick *et al*: Plant Cell Reports (1986) **5**: p81-84.

Tomato seeds (variety Ailsa craig) are first washed in 70% ethanol and then held for sterilization in 10% Domestos for 3 hours before final rinsing and overnight steeping in sterile water at 28°C. Seeds are then germinated in sterile transparent tubs containing germination medium, at 25°C and 16/24 hours photoperiod. Cotyledonary leaves are ready for transformation after 7-10 days and are prepared for transformation by careful transverse cutting so as to yield 2 segments of 0.5-1.0 cm per cotyledon. Explants are pre-incubated for 8 hours on sterile filter papers on a feederlayer of cultured tobacco cells. A culture of the said Agrobacterium strain is resuspended in MS medium plus 3% sucrose to an OD_{590} of 0.4-0.5 and the explants are immersed in this before being returned to the feeder layers and left to co-cultivate for 40 hours.

Explants are then transferred to the surface of an agar selection and counterselection medium containing cefataxine at 500 g/ml (counterselection against Agrobacterium) and kanamycin at 100 g/ml (selection of transformed plant cells) in petri dishes. Explants are thus cultured and transferred to fresh medium at two weekly intervals. As shooting commences the explants are transferred to glass culture vessels and as the shoots expand they are dissected from the original plant and placed on rooting medium containing half the original levels of the antibiotic selections. Shooted and rooted plantlets are grown on and transferred to soil or other growing media following standard horticultural methods with care taken to provide humid conditions during hardening.

Such plants, once cultured, possess increased levels of ascorbic acid in their tissues. This over-production of ascorbic acid may provide an increase in plant yield under stressful conditions. Such plants with elevated levels of ascorbate may also produce fruit or vegetables of increased nutritional quality due to the increased intracellular level of ascorbic acid.

SEQ. 1

Alanine-glutamate-leucine-arginine-glutamate-leucine-glycine-arginine-threonine-glycine-leucine-lysine-leucine-glycine-leucine-valine-glycine-phenylalanine-glycine

Claims

1. Isolated L-galactose dehydrogenase.

2. A polypeptide comprising at least a portion of the amino acid sequence of SEQ1 or functional equivalents to that sequence, or portions of that sequence, which catalyse the conversion of L-galactose to L-galatonolactone by virtue of the degeneracy of the genetic code.

3. Isolated L-galactose dehydrogenase or a polypeptide according to claim 2 wherein the polypeptide is L-galactose dehydrogenase.

4. Isolated L-galactose dehydrogenase or a polypeptide according to claim 2 or 3 wherein the L-galatose dehydrogenase is NAD(P)-dependent.

5. Isolated L-galactose dehydrogenase or a polypeptide according to any one of claims 2 to 4 wherein the catalytic conversion is an oxidation reaction.

6. Isolated L-galactose dehydrogenase according to claim 1 or a polypeptide according to claim 5 wherein the oxidation reaction involves oxidation at C1.

7. Isolated L-galactose dehydrogenase or a polypeptide according to any one of claims 2 to 6 wherein said conversion occurs in plants.

8. A DNA sequence encoding L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7.

9. An organism engineered to express L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7 by incorporation of the DNA sequence according to claim 8 within a gene expression control system

10. An organism according to claim 9 wherein the whole or any part of the organism overexpresses the polypeptide.

11. An organism according to claim 9 or claim 10 wherein the DNA sequence encodes a polypeptide conferring increased tolerance to environmental stresses including those causing oxidative stress.

12. A probe comprising at least a portion of L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7.

13. A probe comprising at least a portion of the DNA sequence according to claim 8 or equivalents to that sequence or portions of that sequence which encode L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7.

14. The production of L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7 by chemical or biological means.

15. Diagnostic tests, assays or monitoring methods using the whole or part of L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7.

16. Diagnostic tests, assays or monitoring methods using a probe according to claim 15.

17. Diagnostic tests, assays or monitoring methods according to either of claims 15 or 16 wherein the tests, assays or monitoring methods comprise microbiological, animal cell or biodiagnostic tests, assays and monitoring methods.

18. An isolated multi-enzyme pathway or method of producing L-ascorbic acid wherein one of the steps of the pathway is catalysed by a L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7.

19. A multi-enzyme pathway or method of producing ascorbic acid according to claim 18 wherein the pathway includes any or all of hexokinase, glucose (hexose)-phospate isomerase, phosphomannose isomerase,phosphomannose mutase,GDP-D-mannose pyrophosphorylase, GDP-D-mannose-3,5-epimerase, GDP L-galactose pyrophosphorylase, GDP-L-galactose phosphorylase, L-galactose-1-phosphate phosphatase, L-galactose dehydrogenase, and L galactono 1-4-lactone dehydrogenase.

20. L-Ascorbic acid produced by the pathway according to either of claims 18 or 19.

21. A compound produced as an intermediate of the pathway according to either of claims 18 or 19.

22. A compound obtained from a precursor compound wherein the precursor compound is produced by the pathway according to either of claims 18 or 19.

23. A dietary supplement comprising ascorbic acid according to claim 20.

24. An organism wherein the whole or any part of the organism contains increased levels of ascorbic acid according to claim 20.

25. An organism engineered to operate a multi-enzyme pathway according to claim 18 or 19.

26. An organism according to claim 24 or 25 engineered to overexpress one or more of the enzymes in the pathway.

27. An organism according to any of claims 24 to 26 which is engineered to express increased levels of L-ascorbic acid by decreasing the activity of at least one enzyme involved in a pathway which diverts carbon away from an ascorbic acid biosynthesis pathway.

28. An organism according to claim 27 in which the said at least one enzyme is involved in polysaccharide or glycoprotein synthesis.

29. An organism according to claim 28 in which the enzyme is a glycosyltransferase.

30. An organism according to claim 28 in which the enzyme is a mannosyltransferase.

31. A method of producing an organism according to claim 27, 28,29 or 30 in which the said at least one enzyme is downregulated by means of expression of antisense copies of DNA sequences encoding the said at least one enzyme.

32. A method according to claim 31 in which the expression of an antisense DNA sequence is controlled by an tissue or organ specific promoter.

33. A plant produced by a method according to any one of claims 31 to 32.

34. A plant according to claim 33 having enhanced tolerance to environmental stress.

35. A plant according to claim 33 or 34 comprising an antisense copy of L-galactose dehydrogenase.

36. An organism according to any of claims 9 to 11 and 24 to 30 wherein the organism is a plant.

37. An organism according to claim 36 wherein the plant is selected from *Arabidopsis thaliana*, *Lycopersicon esculentum* and *Lycopersicon tuberosum*.

38. An organism according to any of claims 9 to 11 and 24 to 30 wherein the organism is a bacterium, yeast or other fungi, plants algae or animal.

39. A method for the non-microbial production of L-ascorbic acid using a plant engineered to express a non-native L-galactose dehydrogenase.

40. A method for the microbial production of L-ascorbic acid using a microorganism engineered to express a non-native L-galactose dehydrogenase.

41. Asorbic acid produced by a method according to claim 39 or 40.

42. A herbicidal composition comprising a compound which inhibits L-galactose dehydrogenase.

43. A herbidcidal composition according to claim 42 in which the compound inhibits expression of L-galactose dehydrogenase or a reaction catalysed by L-galactose dehydrogenase.

44. A method of isolating compounds suitable for use in the herbicidal composition of claim 42 or 43, the method comprising determing the effect of test compounds on L-galactose dehydrogenase activity *in vitro*.

45. A method according to claim 44 in which the L-galactose dehydrogenase is at least partially purified.

46. A method of isolating compounds suitable for use in the composition of claim 42 or 43, the method comprising determing the effect of test compounds on L-galactose dehydrogenase activity *in vivo*.

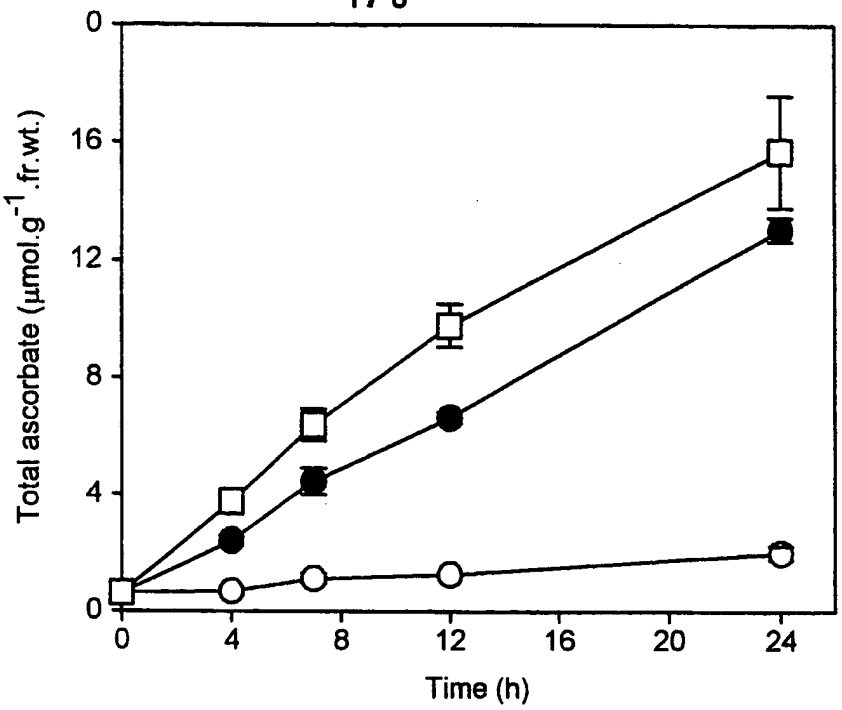
47. A method according to claim 46 in which L-galactose dehydrogenase activity is determined by measurement of ascorbic acid synthesis.

48. A DNA sequence which encodes a form of L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7 which is resistant to the herbicidal composition of claim 42 or 43.

49. A method for generating a plant which is resistant to the herbicidal composition of claim 42 or 43, the method comprising transforming the plant with a DNA sequence according to claim 48 whereby a herbicidal resistant form of L-galactose dehydrogenase or a polypeptide according to any of claims 2 to 7 is expressed.

50. Plants obtained by a method according to claim 49 and reproducible matter derived therefrom.

51. A herbicidal method comprising contacting a plant to be killed with a herbicidal composition according to claim 42 or 43.



Leaf segments were floated on water (○), 25mM L-galactose (●) or 25mM L-galactono-1, 4-lactone (□)

FIG. 1

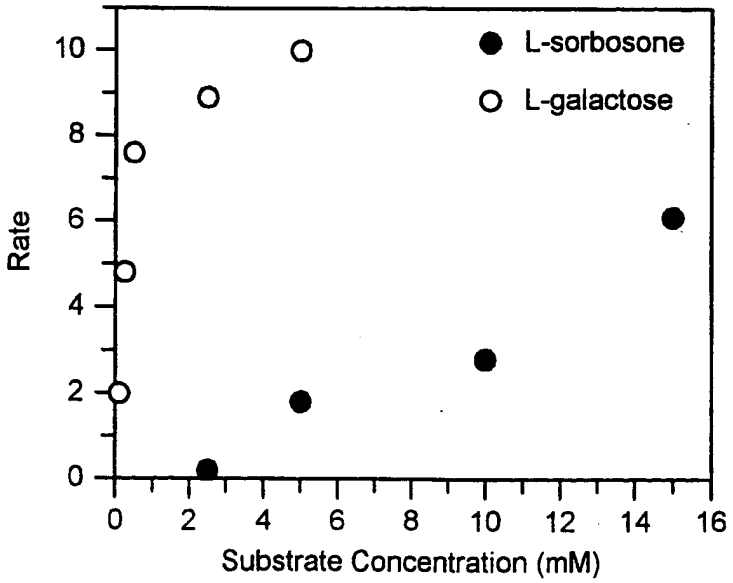


FIG. 2

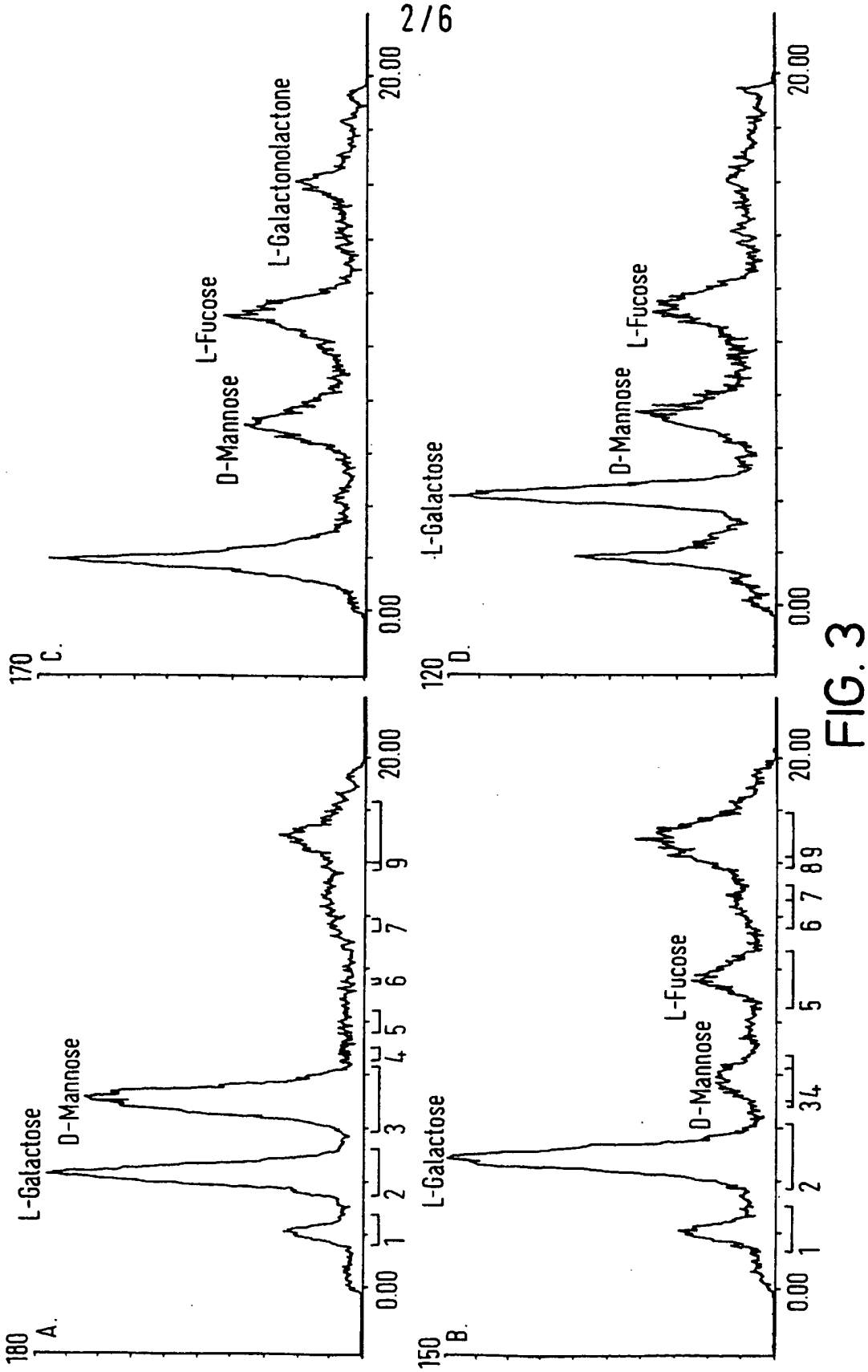

180
A.
L-Galactose
D-Mannose
0.00
20.00
150
B.
L-Galactose
D-Mannose
L-Fucose
0.00
20.00
170
C.
D-Mannose
L-Fucose
L-Galactonolactone
0.00
20.00
120
D.
L-Galactose
D-Mannose
L-Fucose
0.00
20.00

FIG. 3

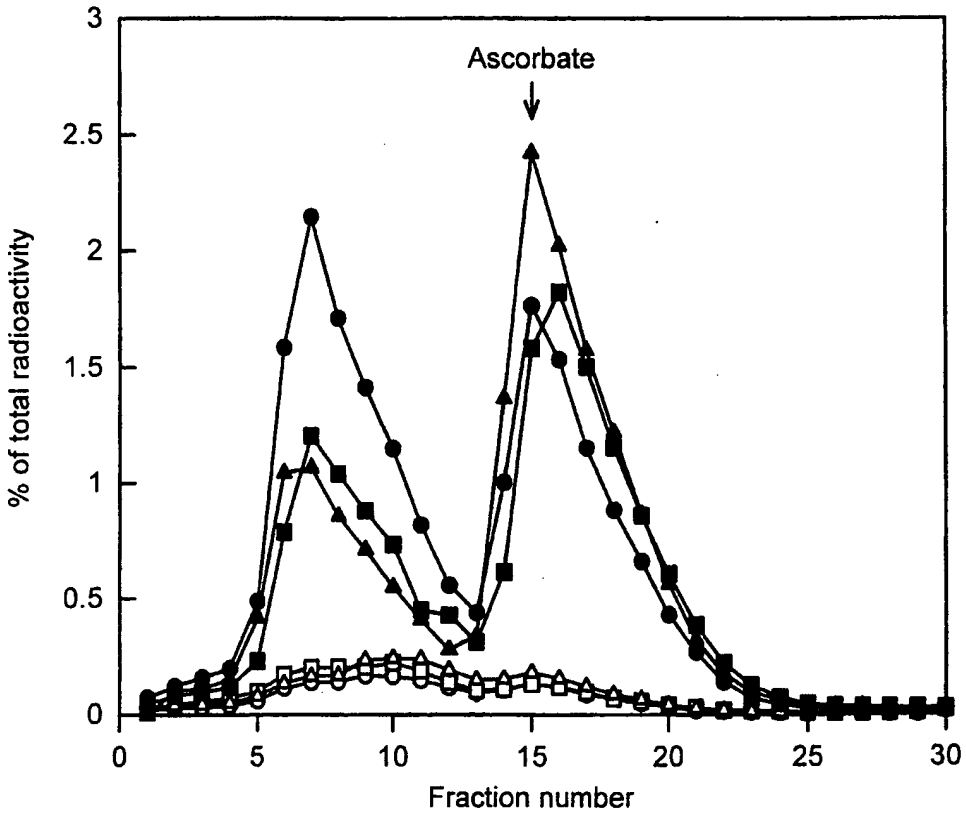
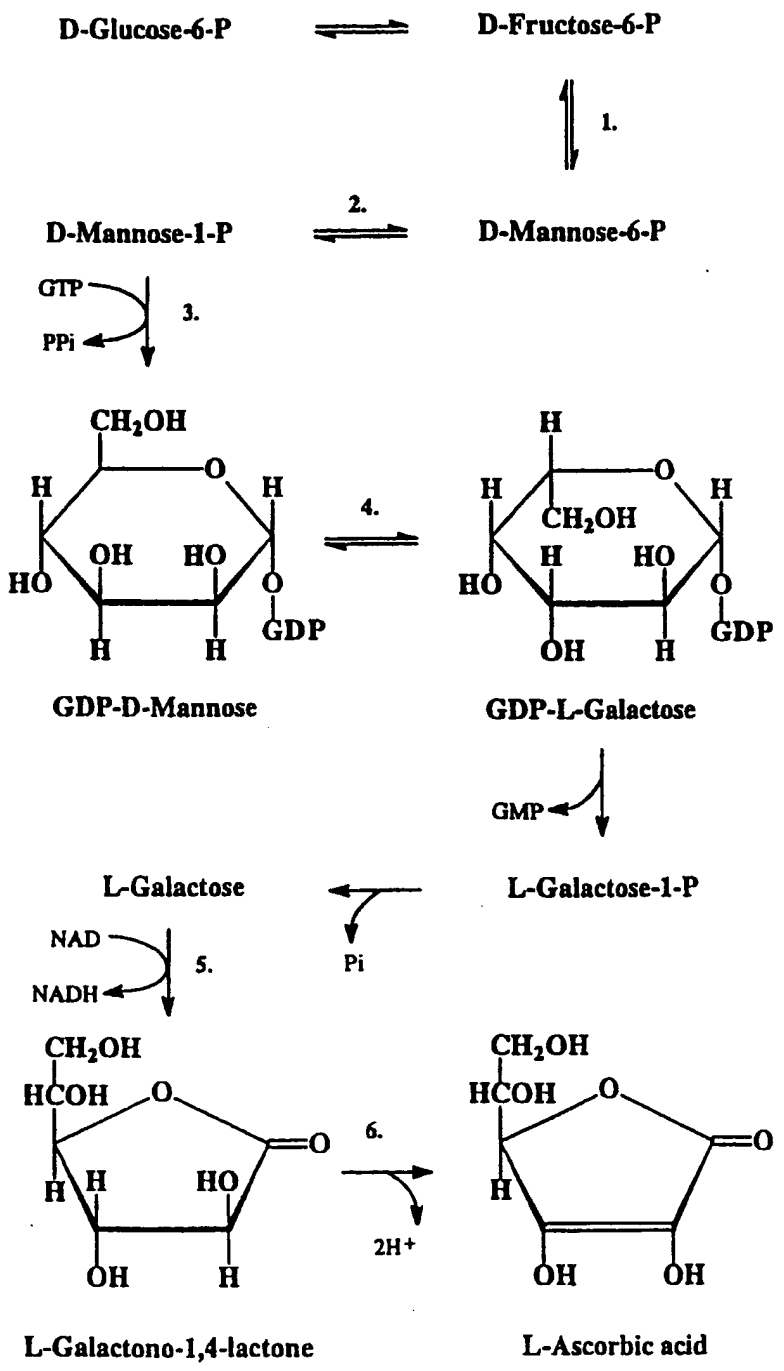

Ascorbate
% of total radioactivity
Fraction number
0
0.5
1
1.5
2
2.5
3
0
5
10
15
20
25
30

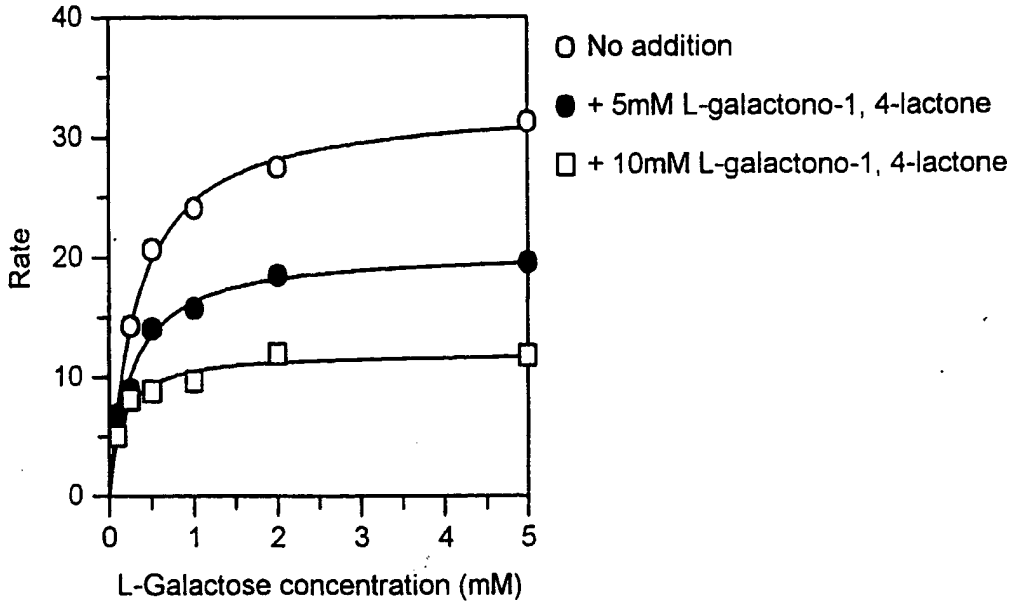
FIG. 4



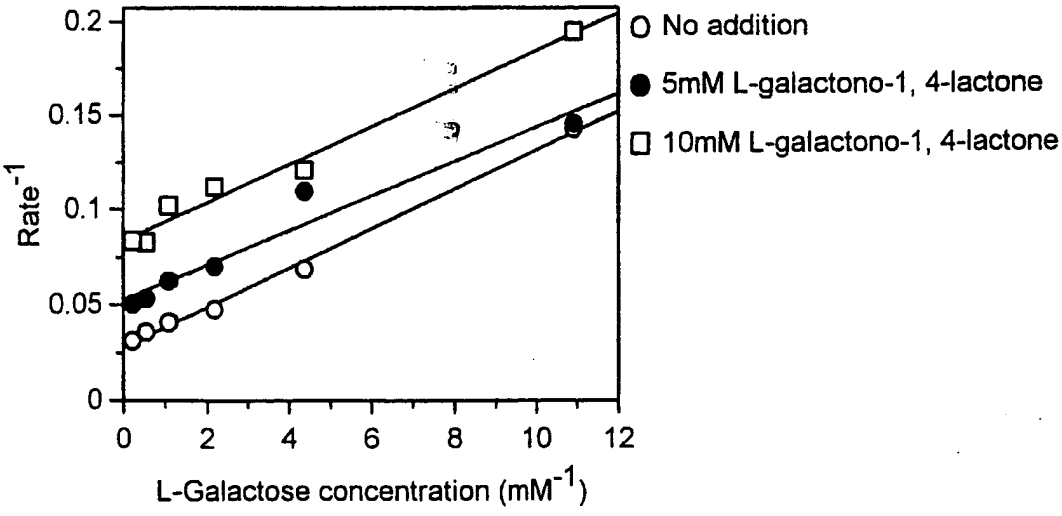
Enzyme names:

1. Phosphomannose isomerase
2. Phosphomannose mutase
3. GDP-D-mannose pyrophosphorylase
4. GDP-D-mannose-3, 5-epimerase
5. L-galactose dehydrogenase
6. L-galactono-1, 4-lactone dehdrogenase

FIG. 5



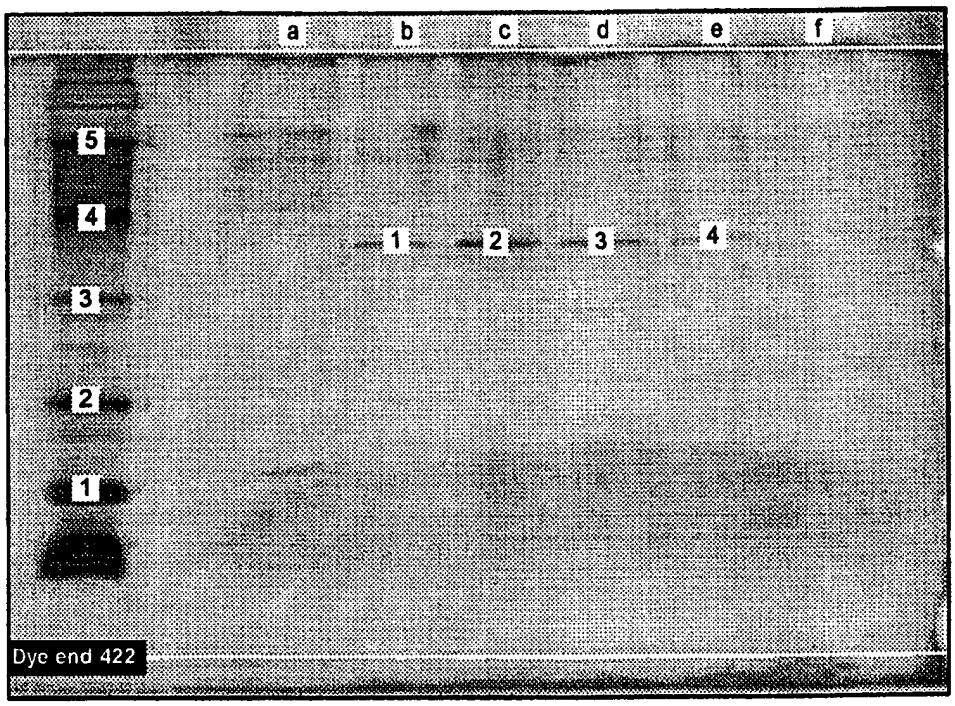
The effect of L-galactono-1, 4-lactone on L-galactose dehydrogenase activity. 1 day old pea embryonic axis enzyme (50-75% ammonium sulphate fraction) was assayed at pH7.5 (50 mM tris) with 0.1 mM NAD. 10 µl extract, assay time: 5 min. No addition: Km 0.33 mM, Vmax 32.8. 5 mM lactone: Km 0.26 mM, Vmax 20.5. 10 mM lactone: Km 0.14, Vmax 12.



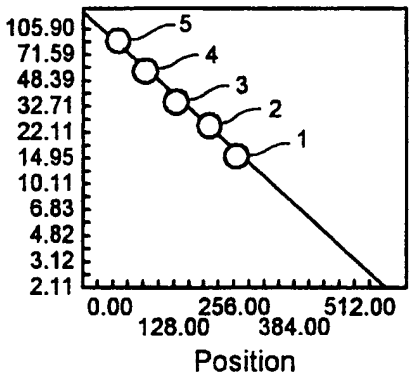
Lineweaver-Burk plot. No addition: Km 0.39, Vmax 35.7. 5 mM lactone: Km 0.19, Vmax 18.9. 10 mM lactone: Km 0.13, Vmax 11.8.

FIG. 6

A)



B)



C)

MARKERS			
Band	Position	Mol. Wt.	RF
1	311	14.40	0.721
2	252	21.50	0.573
3	183	31.00	0.399
4	128	45.00	0.261
5	80	66.20	0.141

QUERIES			
Band	Position	Mol. Wt.	RF
1	150	40.31	0.317
2	150	40.31	0.317
3	152	39.79	0.322

SDS-PAGE analysis of 1ml fractions eluting from Superose 12 gel filtration column.
a) The gel displays a major band at 39.8 kD which coincides with the L-galactose dehydrogenase activity of the fractions. Activities are 0, 6.17, 23.4, 27.8, 6.99 and 0 mAbs/min for samples a-f which eluted after 45, 49, 52, 57, 64 and 70 ml respectively.
b) Calibration curve based on migration of five proteins standards in left hand lane. Standards are lysozyme (14.4kD), soybean tryspin inhibitor (21.5), carbonic anhydrase (31), ovalbumin (45) and bovine serum albumin (66.2).
c) Quantitation of protein size for bands 1-3, corresponding to fractions b-d, based upon band migration relative to protein standards.

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No
PC GB 98/03903

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12N15/52 C12N9/04 C12Q1/68
C12Q1/32 C12P17/04 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LOEWUS M W ET AL: "Conversion of L-sorbosone to L-ascorbic acid by a NADP-dependent dehydrogenase in bean and spinach leaf" PLANT PHYSIOLOGY, vol. 94, 1990, pages 1492-1495, XP002101863 cited in the application see the whole document ---	1-7, 14-17
X	WO 97 04100 A (FRAUNHOFER GES FORSCHUNG ;WISSLER JOSEF (DE)) 6 February 1997 see the whole document ---	20-23,41
A	WO 93 11244 A (WEYERHAEUSER CO) 10 June 1993 see SEQ ID NO: 8 (p. 80-83) see the whole document ---	2
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 May 1999	25.05.99
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Oderwald, H

2

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
P. /GB 98/03903

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WHEELER G L ET AL.: "The biosynthetic pathway of vitamin C in higher plants" NATURE, vol. 393, 28 May 1998, pages 365-369, XP002101864 see the whole document	1-7, 14-23

2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/03903

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 42, 43 and 51 have not been searched due to the lack of sufficient characterization of the claimed compounds.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
P /GB 98/03903

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9704100 A	06-02-1997	AU 6731796 A	18-02-1997
		DE 19628882 A	27-02-1997
WO 9311244 A	10-06-1993	EP 0620852 A	26-10-1994
		JP 7501450 T	16-02-1995
		US 5759828 A	02-06-1998